REPORT DOCUMENTATION PAGE

Form Approved
OMB No 0704-0188

AD-A243 601

REPORT DATE	3. REPORT TYPE AND DATES COVERED
	THESIS/[redacted]

Enzymatic Determination of Phosphatidylglycerol in the 10,000 X g Pellet of Amniotic Fluid

5. FUNDING NUMBERS

6. AUTHOR(S)

George Wendell Jones, Captain

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

AFIT Student Attending: University of Utah

8. PERFORMING ORGANIZATION REPORT NUMBER

AFIT/CI/CIA-91-088

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

AFIT/CI
Wright-Patterson AFB OH 45433-6583

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for Public Release IAW 190-1
Distributed Unlimited
ERNEST A. HAYGOOD, Captain, USAF
Executive Officer

12b. DISTRIBUTION CODE

13. ABSTRACT (*Maximum 200 words*)

14. SUBJECT TERMS

15. NUMBER OF PAGES
46

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT

ABSTRACT

When present in amniotic fluid, phosphatidylglycerol almost always indicates maturity of the fetal lungs. Enzymatic analysis can improve detection sensitivity by measuring the concentration of phosphatidylglycerol in amniotic fluid. By centrifuging (10,000 x g) amniotic fluid to obtain a lamellar body pellet, the phosphatidylglycerol content of the pellet can be determined and compared to established indicators of fetal lung maturity. To enhance the detection of phosphatidylglycerol, lamellar body pellets are reconstituted with less buffer than removed fluid to improve the sensitivity of enzymatic testing. During the study, 128 amniotic fluids were evaluated by comparing the lecithin/sphingomyelin ratio, fluorescence polarization, extracted enzymatic phosphatidylglycerol, and centrifuged (10,000 x g) enzymatic phosphatidylglycerol. The lecithin/sphingomyelin ratio (n = 101) and fluorescence polarization (n = 128) showed correlations of 0.736 and -0.742 respectively. Enzymatic phosphatidylglycerol determinations with phospholipid separation by both centrifugation and extraction showed a correlation of 0.990 (n = 55). Recovery studies demonstrated the lamellar body pellet contains > 97.4% of the phosphatidylglycerol in amniotic fluid. Also, reproducibility of three pooled amniotic fluid controls run 20 times over a 9 day period showed no significant variability between samples either daily or in total. Between run coefficient of variation (CV) for 20 replicates were 27.5%, 5.7%, and 2.6% for controls with means of 0.32, 3.93, and 10.74 µmol/L respectively. The enzymatic procedure was not significantly affected by blood, meconium, bilirubin, or other phospholipids. The results show enzymatic testing of the 10,000 x g pellet represents actual phosphatidylglycerol concentration and thus should be useful in predicting fetal lung maturity.

ENZYMATIC DETERMINATION OF PHOSPHATIDYLGLYCEROL IN THE 10,000 x g PELLET OF AMNIOTIC FLUID

by

George Wendell Jones

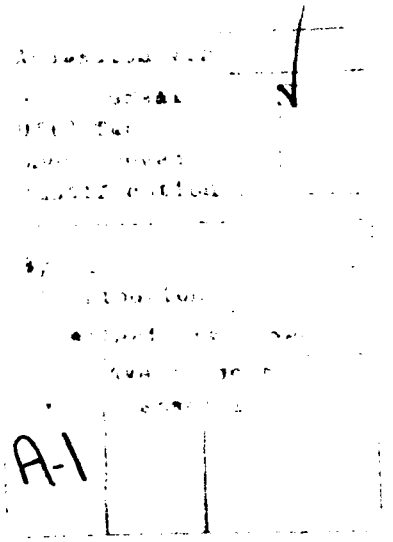
Captain, United States Air Force

1991

46 pages

Master of Science, Medical Laboratory Science

Department of Pathology, University of Utah



ENZYMATIC DETERMINATION OF PHOSPHATIDYLGLYCEROL IN THE 10,000 x g PELLET OF AMNIOTIC FLUID

by

George Wendell Jones

A thesis submitted to the faculty of
The University of Utah
in partial fulfillment of the requirements for the degree of

Master of Science

in

Medical Laboratory Science

Department of Pathology

The University of Utah

August 1991

To Kathy, Brittney, and Joshua:

Thank you for tolerating late nights, early mornings, and postponed family outings. Your support, patience, and understanding made this thesis possible.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGMENTS

With the help of many individuals, completion of this thesis was possible. While mentioning everyone who helped along the way would be difficult, there are a few that deserve special recognition:

Edward R. Ashwood, M.D. for encouragement, suggestions, support, and critical reviews of my work.

Stephen E. Palmer for assistance with the computers and helping find answers to countless questions.

James T. Wu, Ph.D and Michael W. Varner, M.D. for reading and critiquing the manuscript.

Special thanks to the chemistry departments at the Associated Regional and University Pathologists, Research Park Laboratory for the use of analytical equipment and technical support. Also, to the United States Air Force, without their sponsorship, completion of a master's degree in medical laboratory science would not have been possible.

INTRODUCTION

Functional Neonatal Pulmonary Maturity and Immaturity

Maturity of the fetal lungs is a major concern of physicians dealing either with pregnant women in premature labor or their prematurely delivered infants. This concern stems from the association between progressive prematurity and functional neonatal pulmonary immaturity. Functional neonatal pulmonary maturity is caused by alterations in fetal pulmonary physiology or in the transitional physiology during and just after birth (1). Functional neonatal pulmonary immaturity is a general term describing any condition that adversley affects the exchange of pulmonary gases. Some specific eponyms are: transitional respiratory distress, transient tachypnea of the newborn, hyaline membrane disease, aspiration syndromes, air-leak syndromes, persistent pulmonary hypertension, and pulmonary hypoplasia (1). The most precise is hyaline membrane disease which requires histological review for a definitive diagnosis, but represents the extreme endpoint of functional immaturity caused by inadequate amounts of pulmonary surfactant.

Without adequate or functional surfactant, the lung's ability to maintian a stable environment and equilibrium of pressure between alveolar spaces is impaired. Development of the disease starts with focal atelectasis and formation of a hyaline membrane in the bronchiole as the epithelium is separated from the basement membrane. As the formation of the membrane progresses, the lungs become congested with fluid and trapped air that inhibit the ability of the infant to breathe (2). If untreated, the disorder may be fatal.

Prematurity is necessary for an unequivocal diagnosis of hyaline membrane disease but

is not a guarantee the disease will be present. With approximately 300,000 premature births each year, only 40,000 infants will develop hyaline membrane disease (2). Table 1 shows how the incidence of disease increases as gestational age decreases. This incidence correlates with the production of surfactant phospholipids that indicate maturity of the fetal lungs.

Pulmonary surfactant is the material synthesized by the granular (type II) pneumocytes in the lungs. Surfactant reduces alveolar surface tension during expiration and prevents collapse of the lung. It is a complex mixture of protein, phospholipid and neutral lipid (2). About 78% of the phospholipid in the term newborn is composed of phosphatidylcholine (PC), 9% phosphatidylglycerol (PG), and 4% phosphatidylinositol (PI) (3). The remaining 9% is composed primarily of phosphatidylethanolamine (PE), phosphatidylserine (PS), and sphingomyelin (3).

Within the granular pneumocytes, the surfactant is synthesized in the rough and smooth endoplasmic reticulum membranes. The synthesized phospholipids are transported to the Golgi apparatus and transformed into lamellar bodies. These lamellar bodies consist of concentric layers of phospholipid membrane with a central core and outer coat of protein. Upon exocytosis of the lamellar bodies, the concentric layers unfold to form a lattice structure called tubular myelin. This lattice structure contains the surfactant which reduces

Table 1. Incidence of hyaline membrane disease (HMD)

Gestation (Completed Weeks)	Incidence of HMD %
37 - 40	.9
35 - 36	5.4
33 - 34	20.5
31 - 32	35.0
29 - 30	64.3

surface tension (2). The ability of the surfactant to effectively reduce surface tension and prevent atelectasis is dependent on the composition and concentration of phospholipid.

Many researchers have studied the ability of the surfactant to lower surface tension (3), and have found that the phospholipid composition was critical to the surfactant's ability to reduce surface tension. While phosphatidylcholine was known to be the primary component of the surfactant, in vitro studies using a Wilhelmy balance showed that, by omitting the minor phospholipids, surfactant function is impaired. In one study (3) where purified phosphatidylcholine was applied to an aqueous subphase, it did not spread into a surface-active film at temperatures below 41°C. However, when phosphatidylglycerol was added prior to application, the mixture spread into a surface-active film at physiological temperatures, suggesting that the minor phospholipids may be necessary for normal surfactant function in the mature lung.

Indicators of Fetal Lung Maturity

For 20 years, the lecithin/sphingomyelin ratio (L/S) has been the "gold standard" (4) for evaluating fetal pulmonary maturity . Developed by Gluck et al. (5), the lecithin/sphingomyelin ratio compares the amniotic fluid concentration of lecithin (phosphatidylcholine) to sphingomyelin through the use of thin-layer chromatography (TLC). The amount of lecithin and sphingomyelin separated by TLC are compared. When the lecithin concentration in the sample is at least twice that of sphingomyelin, the sample is considered positive for fetal lung maturity. Around the 33rd week of gestation, the lecithin concentration begins to increase and remains elevated through term while the sphingomyelin concentration remains relatively constant. This increase occurs a few weeks before the production and inclusion of phosphatidylglycerol in the surfactant (as judged by current phosphatidylglycerol detection methods).

After Gluck's initial description of this phenomenon, many reports were published that validated his findings. In reports by Spillman et al. (6) and Chapman et al. (4), the

predictive value of fetal lung maturity was determined for 239 and 144 cases respectively where the lecithin/sphingomyelin ratio was ≥ 2.0. Spillman et al. (6) showed that the lecithin/sphingomyelin ratio correctly predicted mature lungs in 95% of cases and Chapman et al. (4) reported 98.6% of cases correctly predicted. The ability to predict immaturity was not as accurate. In both studies, only 50% of the cases reported as immature had respiratory difficulty following birth. This inability to accurately predict immaturity is not limited to the lecithin/sphingomyelin ratio, most lung maturity tests have the same problem.

Phosphatidylglycerol plays a vital role in the function of the surfactant. Like phosphatidylcholine, synthesis of phosphatidylglycerol begins late in gestation. Hallman et al. (7) examined 66 amniotic fluids and demonstrated that phosphatidylglycerol was detectable beginning about the 35th week of gestation and increased rapidly through term. The fact that detection of phosphatidylglycerol in the surfactant is indicative of fetal lung maturity has been well-documented (4,6,8-10). When phosphatidylglycerol was detected, Hamilton et al. (11) reported that 99% of the cases had functionally mature lungs and Spillman et al. (6) reported 96% with functionally mature lungs.

Detecting phosphatidylglycerol in amniotic fluid can be performed by numerous methods. Some of the more common methods are: one- and two-dimensional thin-layer chromatography (3,12), enzymatic analysis of amniotic fluid and phospholipids extracted from amniotic fluid (13,14), and immunologic agglutination (4,8). Thin-layer chromatography is the most common method used. It uses the principle that each phospholipid migrates at a different rate on a chromatography gel. Both one- and two-dimensional chromatography are able to identify phosphatidylglycerol, but two-dimensional improves the resolution and allows better detection. Thin-layer chromatography is limited by problems in quantitating the results and difficulty with interpretation (4).

Enzymatic analysis corrects some of the problems associated with thin-layer chromatography by providing a quantitative result requiring little interpretation. Using an

enzymatic reaction sequence to measure phosphatidylglycerol, most procedures can detect concentrations of ≥ 0.5 μmol/L, with published maturity cut-offs being 1.5 and 2.5 μmol/L (9,15). Enzymatic procedures differ primarily on the type of specimen used. PG-Numeric™ (15), the only commercially available procedure, uses amniotic fluid without phospholipid separation. Using untreated amniotic fluid simplifies the procedure, but the glycerol content of the amniotic fluid greatly increases the background absorbance making it difficult to accurately measure small concentrations. Other procedures use chloroform:methanol extraction to remove the glycerol from the amniotic fluid. By separating the phospholipids a higher quality specimen is obtained that does not contain glycerol (13). The improvement of the specimen comes with a slight loss of phospholipids and increase of time involved and technical difficulty. While not extensively used, enzymatic determinations show the most promise for a quick, simple, and accurate method for detecting phosphatidylglycerol.

The fastest method for phosphatidylglycerol is the Amniostat-FLM™ slide agglutination test. With an antiserum specific for phosphatidylglycerol (4), the Amniostat-FLM™ utilizes an antigen-antibody agglutination reaction to identify phosphatidylglycerol in amniotic fluid. While quick and simple to perform, it lacks an ability to detect low concentrations and is subject to interpretations of agglutination patterns in the reaction. Regardless of the method used, all are comparable in the ability to detect phosphatidylglycerol (4,8). In deciding which method to use, it is the trade-offs between time, sensitivity, accuracy, and difficulty that must be considered.

While both the lecithin/sphingomyelin ratio and phosphatidylglycerol determinations are very predictive of fetal lung maturity, phosphatidylglycerol has advantages over the lecithin/sphingomyelin ratio. As described by Strassner et al. (16), the detection of phosphatidylglycerol is not hindered by contamination with blood (cells or plasma). Also, a review by Spillman and Cotton (3) stated that neither meconium, vaginal secretion, or blood effected the results of phosphatidylglycerol determinations. In addition, some

phosphatidylglycerol assays are easier and quicker to perform than are lecithin/sphingomyelin assays. Thus, phosphatidylglycerol is worthy of consideration when choosing tests for prediction of fetal lung maturity.

Proper development of the fetal lungs is vital to the health of the newborn. When lung development is delayed or the infant is born prematurely, the newborn's chance of having functional neonatal pulmonary immaturity is greatly increased. While not all problems can be identified prior to birth, the maturity of the lung can be determined and does correlate with other complications of prematurity such as: intraventricular hemorrhage, patent ductus arteriosus and hypoglycemia. The ability to determine lung maturity status is an important tool when dealing with complicated pregnancies and premature labor.

Phosphatidylglycerol is capable of accurately predicting fetal lung maturity. Being unaffected by contaminates that render the lecithin/sphingomyelin ratio invalid, phosphatidylglycerol determinations can be used in a variety of situations. The development of nonchromatographic methods also reduces difficulty and time involved. Phosphatidylglycerol is quite specific for lung maturity (95.0 - 98.6%) (4,6) and false positives are rare except in situations resulting from bacterial contamination of vaginal pool amniotic fluids (17,18). With careful management of amniotic fluid and proper method development, phosphatidylglycerol could become a replacement for the lecithin/sphingomyelin ratio.

STUDY OVERVIEW

The purpose of this study is to develop simplified and improved procedures for the determination of phosphatidylglycerol concentrations in amniotic fluid. To do this, the study is divided into three parts. First, an alternative to the labor-intensive extraction method will be evaluated for separating phospholipids from amniotic fluid. Second, modification to the enzymatic reaction sequence shown in Figure 1 is introduced to enhance sensitivity. The third phase of the study is designed to compare the quantitative results of the enzymatic testing with established clinical laboratory tests for evaluating fetal lung maturity: lecithin/sphingomyelin ratio, fluorescence polarization, and phosphatidylglycerol determined by thin-layer chromatography.

Of the areas addressed by this study, the separation of phospholipids from amniotic fluid should result in the most significant change from current methods. Extraction by addition of a chloroform:methanol solution (9,13,14) or filtration (15) are the current methods of amniotic fluid preparation for enzymatic testing. While both provide a suitable testing matrix for the enzymatic procedure, problems with phospholipid recovery, technical

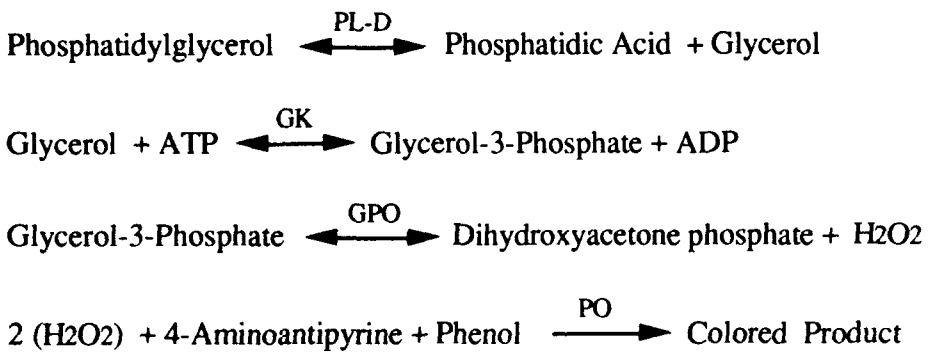


Figure 1. Enzymatic reaction sequence.

difficulty, or removal of endogenous glycerol make these undesirable for use. To solve these problems, separation by high speed centrifugation (10,000 x g) will be incorporated into the enzymatic method. Centrifugation has shown an ability to provide a pellet of surfactant rich phospholipids (12,19,20). Enzymatic testing of the pellet should simplify the measurement of phosphatidylglycerol and improve lung maturity predictions.

Concentration of the phospholipids is another unique modification that should provide better maturity predictions in transitional specimens. Depending on the reference method used (9,15), phosphatidylglycerol concentrations of 1.5 or 2.5 μmol/L indicates lung maturity. Only a very sensitive method can measure such low concentrations of phosphatidylglycerol. By concentrating the phospholipids, the minimum detectable concentration is improved, allowing better differentiation between mature and immature amniotic fluid specimens.

The combination of phospholipid separation by centrifugation and concentration should be a valuable enhancement to enzymatic testing. This study will demonstrate how the modifications correlate with established procedures for fetal lung maturity.

MATERIALS AND METHODS

Instruments

For preparation of samples by centrifugation, an Eppendorf refrigerated micro centrifuge, model 5402 (Brinkmann Instruments, Inc., Westbury, NY 11590) was used. The centrifuge was equipped with an 18 position, 45° fixed angle rotor (P/N F-45-18-11) and had an operating range of 1,000 to 14,000 rpm (80 to 15,800 x g) adjustable in steps of 100 rpm. The refrigeration unit could maintain a centrifuge temperature in the range of –9 to +40°C. Disposable 1.5 mL, 39 x 10 mm conical centrifuge tubes (Sarstedt, Germany, P/N 72.690) were used.

Absorbances were measured with a Beckman DU-70 Spectrophotometer (Beckman Instruments, Inc., Scientific Instruments Division, Fullerton, CA 92631-3100). This instrument is a high speed, microprocessor controlled spectrophotometer operating in the wavelength range of 190 - 900 nm. The instrument operates in either a single, dual, or multiple wavelength mode in addition to wavelength and time drive scans. Wavelength accuracy is ± 0.5 nm and absorbance accuracy is ± 5%. The instrument was equipped with a peltier temperature controller (Beckman, P/N 523422) capable of maintaining temperatures between 15 and 40°C. For this study, an Auto 6-Sampler accessory (P/N 523409) was used in place of the sipper accessory with flowcell (P/N 523404) available on the instrument. In place of the flowcell, disposable 10 x 4 x 45 mm cuvettes (Sarstedt, P/N 67.742) were used for measuring absorbances.

One additional centrifuge, a Jouan CT 422 Refrigerated Centrifuge (Jouan, Paris, France) was used in separating the liquid phases during chloroform:methanol extraction of the amniotic fluid. Using an M4 swing out rotor (P/N 11175338) and holders for 15 mL

tubes (P/N 11174208), the centrifuge could operate from 8 to 4160 x g (200 to 4500 rpm) and maintain a temperature between 4 and 60°C.

Reagents

Sources

Chemicals, enzymes, and standards used to perform this study were acquired from three primary sources: Sigma Chemical Co., St. Louis, MO 63178; Boehringer Mannheim Biochemicals, Indianapolis, IN 46250; and Avanti Polar Lipids, Inc., Alabaster, AL 35007.

Phospholipase D (PL-D, from *Streptomyces chromofuscus*, EC 3.1.4.4), glycerol-3-phosphate oxidase (GPO, from *Pediococcus species*, EC 1.1.3.21), peroxidase (PO, from horseradish, EC 1.11.1.7), adenosine triphosphate (ATP), 4-aminoantipyrine (4-AAP), 2-hydroxy 3,5-dichlorobenzene-sulfonate (HDCBS), triton X-100, and glycerol were obtained from Sigma Chemical Co. Glycerokinase (GK, from *Bacillus stearothermophilus*, EC 2.7.1.30) was obtained from Boehringer Mannheim Biochemicals. Phosphatidylglycerol (PG), phosphatidylcholine (PC), phosphatidylinositol (PI), and phosphatidylethanolamine (PE) were obtained from Avanti Polar Lipids.

Preparation

The study used a two step, three reagent system developed by combining the work of Artiss et al. (13) and Muneshige et al. (14). Each reagent, designated PG reagent A, B, and C, was prepared in a 50 mmol/L Tris-HCl buffer, pH 7.6, containing 5 g/L triton X-100. This buffer, designated triton buffer, was used to prepare reagents and standards. PG reagent A was prepared to give the following concentrations per liter of reagent: glycerokinase, 1 kU; glycerol-3-phosphate oxidase, 3 kU; ATP, 2 mmol; calcium chloride, 10 mmol; and magnesium chloride, 8 mmol. PG reagent B was prepared identical to reagent A with the addition of phospholipase D, 80 kU/L. PG reagent C was prepared to give the following concentrations per liter of reagent: peroxidase, 2 kU;

4-aminoantipyrine, 1.5 mmol; and HDCBS, 3 mmol. All reagents were stored protected from light. Stability was at least 12 hours when stored at 4°C and 21 days when stored at less than –20°C.

Phospholipid standards were prepared in triton buffer and stored at 4°C for up to 14 days or at less than –20°C for up to 30 days. Phosphatidylglycerol was prepared for linearity standards and as a test solution for initial method development. Ten standards of phosphatidylglycerol were prepared ranging from 0 to 500 µmol/L. Phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol were all prepared at a concentration of 200 µmol/L for interference studies of the proposed enzymatic method.

Procedures

Lecithin/Sphingomyelin Ratio and Phosphatidylglycerol by TLC

Determinations of the lecithin/sphingomyelin (L/S) ratio and qualitative phosphatidylglycerol (PG) were performed at the Associated Regional and University Pathologists (ARUP), Special Chemistry Laboratory, using a thin layer chromatographic method described by Ashwood et al. (21). Amniotic fluid samples were processed in triplicate. Each sample was prepared by extracting 1 mL of amniotic fluid with 1 mL of methanol and 2 mL of chloroform. After mixing and centrifugation of the sample, the lower chloroform phase was removed with a pasteur pipet, placed in a conical 3 mL glass tube, and evaporated to dryness under a stream of air in a 60°C water bath. The sample was then placed in an ice bath and 0.5 mL of ice-cold acetone was added. The acetone was discarded and 10 µL of chloroform was added to dissolve the acetone insoluble precipitate. For the L/S ratio, a 1 x 13 cm strip of thin-layer silica gel (Eastman 13179) was spotted with the entire contents from one tube. The strip was developed in a solution of methanol/water/chloroform (5/0.8/13.5 v/v/v) until the solution migrated 8 cm. Following development, the strip was air dried then stained with ammonium molybdate. The L/S ratio

was calculated by dividing the size of the lecithin spot by the size of the sphingomyelin spot.

To determine the presence of phosphatidylglycerol, the dissolved precipitate from the other two tubes were spotted together on an 8 x 8 cm sheet of thin-layer silica gel. This sheet was then developed in a solution of chloroform/methanol/water (65/25/4 v/v/v) until the solvent migrated to the top of the sheet. Next, the sheet was dried, a phosphatidylglycerol standard spotted on the sheet, and developed in a solution of tetrahydrofuran/methylal/methanol/4N ammonium hydroxide (50/15/10/5.5 v/v/v/v) at a right angle to the first development. Again the sheet was dried then stained with ammonium molybdate to detect phosphatidylglycerol. A positive test resulted from a spot appearing on the sheet parallel to the phosphatidylglycerol standard.

Fluorescence Polarization

Like the lecithin/sphingomyelin ratio and phosphatidylglycerol, fluorescence polarization analyses were performed in the ARUP Special Chemistry Laboratory. The method performed by the laboratory was developed by Tait et al. (22) and used in a series of studies validating its ability to predict fetal lung maturity (22-25). First, the amniotic fluid was centrifuged for 2 minutes at 830 x g (2000 rpm) and the supernate removed. The supernate was then prepared for testing on an Abbott TDx Fluorescence Polarimeter (Abbott Laboratories, Irving, TX 75015). Supernate (0.5 mL) was diluted by adding 1.0 mL of TDx buffer directly into a fluorometer cuvette and mixed gently by repipetting. This was repeated for all specimens and controls. Using the TDx Photo Check procedure, the background fluorescence intensity and polarization was measured. Next, 10 µL of a fluorescent dye, 1-palmitoyl-2{6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl} phosphatidylcholine (NBD-PC), was added to each cuvette, mixed gently, and placed in the instrument to allow temperature equilibration. Again, using the Photo Check procedure, the total fluorescence intensity and polarization was measured approximately

6.5 minutes after addition of the dye. The TDx reports polarization (P) in units of mP (1000 mP = 1 P). Finally, the net polarization was calculated on a personal computer using the following procedure:

(a) Calculate net fluorescence intensity:

$$I_{net} = I_{tot} - I_{bkg}.$$

(b) Convert polarizations (P_{tot}, P_{bkg}) to anisotropies (r_{tot}, r_{bkg}):

$$r = [2P/1000] / [(3-P) / 1000].$$

(c) Calculate net anisotropy (r_{net}):

$$r_{net} = [(r_{tot}I_{tot}) - (r_{bkg}I_{bkg})] / I_{tot}.$$

(d) Convert net anisotropy to net polarization:

$$P_{net} = 1000 [3r_{net} / (2 + r_{net})].$$

Enzymatic Phosphatidylglycerol

Ascertainment of Samples. Amniotic fluids tested using the enzymatic method were aliquots remaining from samples collected for diagnostic fetal lung maturity testing. Use of these samples has been approved by the University of Utah Institutional Review Board (approval # 2132). All samples were centrifuged at 830 x g (2000 rpm) for 2 minutes, tested for lung maturity, then frozen at –60°C. Samples noticeably contaminated with blood, meconium, or vaginal secretions were not included in the enzymatic procedure evaluation. Excluding these samples helped ensure that accurate comparisons with the lecithin/sphingomyelin ratio and fluorescence polarization could be made. While phosphatidylglycerol determinations are unaffected by these contaminates (16,26), studies indicate the lecithin/sphingomyelin ratio and fluorescence polarization are affected (22,27). Prior to performing enzymatic testing, fluids were thawed at room temperature, mixed on a rocker for 30 minutes, and treated for separation of phospholipids as required. Two specimen types were used during the study: chloroform:methanol extracted amniotic fluid,

and amniotic fluid centrifuged at 10,000 x g to form a lamellar body pellet containing fetal lung surfactant.

Chloroform:methanol extraction. Well-mixed amniotic fluid (1.5 mL) was placed in a 15 mL screw top glass tube. Three mL of a chloroform:methanol (2/1 v/v) solution were added and the tube vortexed for two minutes at a low speed. To speed the separation of chloroform from the aqueous methanol phase, the specimen was spun at 3300 x g (4000 rpm) for 5 minutes in the Jouan centrifuge. Then, using a pasteur pipet, the lower chloroform phase was removed and placed in a conical glass tube. The conical tube was incubated in a 37°C water bath and the chloroform evaporated under a stream of air. Once the chloroform was removed, 0.5 mL of triton buffer was added and the tube incubated at 65°C for 5 minutes to resuspend the phospholipids. This resulted in a 3-fold concentration of the extracted phospholipids. Finally, the tube was vortexed on low to ensure complete mixing prior to testing.

Centrifugation at 10,000 x g. Well-mixed amniotic fluid (1.5 mL) was placed in a 1.5 mL conical centrifuge tube and sealed with the attached cap. Samples were then placed into the Eppendorf 5402 micro centrifuge which was cooled to 4°C. The centrifuge was set to spin for 20 minutes at 10,000 x g (11,200 rpm). When centrifugation was complete, the tubes were removed and the supernate decanted from the pellet. Then, while still inverted, the tubes were tapped gently on a paper towel to remove any fluid in the bottom of the tubes. The tubes were left inverted for 5 to 10 minutes, then the mouth of the tubes were blotted with adsorbent paper to remove any residual supernate. Next, 0.5 mL of triton buffer was added to the tube and the tube incubated at 65°C for 5 minutes to resuspend the pellet. This resulted in a 3-fold concentration of the amniotic fluid phospholipids. Finally, the tubes were vortexed on low to ensure complete mixing prior to testing.

Enzymatic procedure. Two tubes were prepared for each sample, control, and standard to be tested, labeled A and B, and 0.2 mL of phospholipid solution was placed in each tube. To tube A was added 0.2 mL of PG reagent A. This tube served as a glycerol and

reagent blank (PG reagent A did not contain phospholipase D). To tube B was added 0.2 mL of PG reagent B. After addition of the appropriate PG reagent A and B, the tubes were mixed by shaking for 5 seconds then incubated in a 37°C water bath for 15 minutes. Next, 0.2 mL of PG reagent C (color development reagent) was added to each tube, shaken for 5 seconds, then incubated at 37°C for 15 minutes. Hydrogen peroxide (H_2O_2) formed during the PG reagent A and B reactions combined with a dye to produce a red chromogen complex that absorbs light at a wavelength of 510 nm. Finally, all tubes were measured for absorbance of light using the Beckman DU-70 spectrophotometer. Using the single wavelength mode set to average 10 readings, samples were placed in 10 x 4 x 45 mm disposable cuvettes and measured against deionized water. The absorbance value from tube A was subtracted from tube B resulting in a net absorbance that was directly proportional to the phosphatidylglycerol content of the amniotic fluid. The net absorbance was compared to a standard curve to determine phosphatidylglycerol concentration.

Phosphatidylglycerol concentration determination. With each set of samples or controls, a three point standard curve was established using aqueous phosphatidylglycerol standards of 0, 30, and 60 µmol/L. Due to the 3-fold concentration of phospholipids from the specimens and the inability to concentrate aqueous standards by centrifugation, the three standards represent amniotic fluid phosphatidylglycerol concentrations of 0, 10, and 20 µmol/L respectively. The standard curve represents the expected physiological range of phosphatidylglycerol. While a new standard curve was prepared for each run, all showed linearity with a typical correlation coefficient of 0.999. The concentration for each sample and control was then calculated from the standard curve line equation and net absorbance. Results are reported as µmol/L of phosphatidylglycerol in amniotic fluid.

RESULTS

Development of Enzymatic Procedure

Chromogen Selection

Developing a reagent that would maximize the sensitivity of the procedure was a major goal. Several oxidative dyes were studied to enhance the absorbance of the reaction. The last step in Figure 1 (page 7) was isolated by using hydrogen peroxide standards and replacing 4-aminoantipyrine with two different dyes: o-diansidine and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid). Each dye was evaluated using the same batch of hydrogen peroxide standards for linearity and stability. The goal was to have a product linear through 100 µmol/L (values were expected to range from 0 to 60 µmol/L) and a stable absorbance for at least 1 hour. This time period would allow adequate time for test completion.

Because of its extensive use in the literature (13,14), 4-aminoantipyrine was evaluated first. Testing started by determining the visible spectrum of the colored product to establish the wavelength that produced maximum absorbance, which was 510 nm. Next, stability was evaluated by measuring the absorbance of the colored product over 60 minutes, on the DU-70 spectrophotometer, The results showed the maximum absorbance was reached at 7 minutes and remained stable through 60 minutes. A reaction time of 15 minutes was selected to ensure complete conversion of the H_2O_2. Finally, the linearity was checked using hydrogen peroxide standards of 0 - 250 µmol/L. The result showed linearity with a correlation coefficient for the regression line being 0.999 and the absorbance of the 125 µmol/L standard was 0.4.

The next dye evaluated was 2,2'-azino-bis(ethylbenzthiazoline-6-sulfonic acid) (azino-bis). Using the same evaluation process as 4-aminoantipyrine, azino-bis showed maximal absorbance at 410 nm, linearity through 125 µmol/L ($r = 0.999$) and questionable stability. Azino-bis showed a significant improvement in absorbance of a 125 µmol/L hydrogen peroxide standard with a result of 2.0. This improvement was quickly overshadowed by the instability of the dye. Testing showed immediate degradation of the colored product after achieving maximum absorbance. It was determined the peroxidase catalyzed the degradation as it could be stopped by the addition of acid to lower the pH from 7.6 to < 4. While the increased absorbance was an advantage, the stability of the colored product raised questions of reproducibility and accuracy.

The final dye tested was o-dianisidine. This dye produced a stable, linear ($r = 1.000$) complex that gave absorbance readings comparable to 4-aminoantipyrine. Absorbance of a 125 µmol/L standard was 0.4 at a wavelength optimum of 450 nm. With a stable colored product through 60 minutes and linearity to 250 µmol/L, o-diansidine was an acceptable alternative to 4-aminoantipyrine.

Of the dyes evaluated, only azino-bis produced the sensitivity desired. Figure 2 shows the improvement over 4-aminoantipyrine and o-diansidine. However, even with the absorbance improvement, azino-bis was not used due to the disadvantage of instability. o-Diansidine was also excluded as it did not demonstrate improvements over 4-aminoantipyrine. So, the choice for the reaction scheme remained 4-aminoantipyrine as it provided linearity, stability, and was supported by published research on phosphatidylglycerol determinations.

Oxidative Substrate Selection

Another modification to the reagent composition was the choice of oxidative substrate used in the final step of the reaction sequence. Hydrogen peroxide oxidizes the dye and oxidative substrate in the presence of peroxidase to form a colored product.

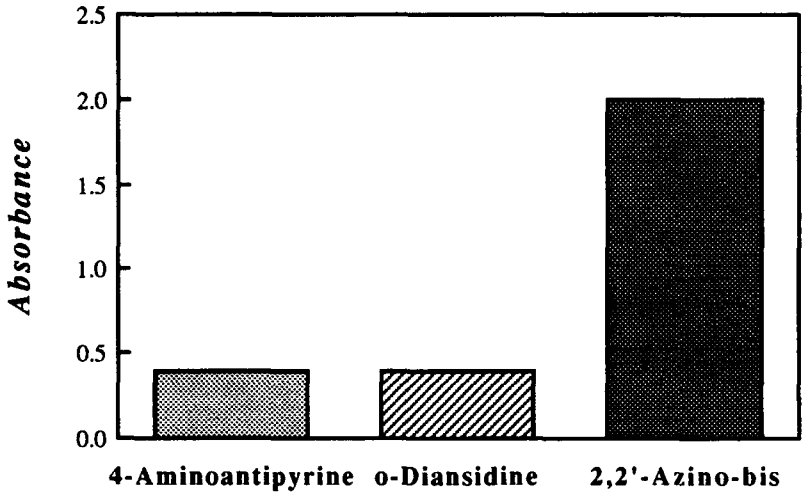


Figure 2. Effect of optional enzymatic dyes in PG reagent on the absorbance of a 125 µmol/L hydrogen peroxide standard.

Having selected a dye, alternatives to the standard oxidative substrate were made to further optimize the reaction. Phenol, used by Muneshige et al.(14), was tested first ; 4-chlorophenol and 2-hydroxy 3,5-dichlorobenzenesulfonate (HDCBS) were also evaluated. Selection of the additional substrates came from review of published literature and test methodologies with similar reaction schemes; 4-chlorophenol was discovered by a review of triglyceride procedures. The final three steps of Boehringer Mannhiem's Triglyceride-GB procedure are identical to the proposed phosphatidylglycerol method with the exception of exchanging 4-chlorophenol for phenol. HDCBS was used by Artiss et al.(13) in an enzymatic phosphatidylglycerol procedure. The evaluation of the three substrates was made by comparing the absorbances of a 125 µmol/L phosphatidylglycerol standard tested with PG reagent containing the various substrates. HDCBS gave a significantly higher absorbance (Figure 3). While all three substrates demonstrated endpoint stability and linearity, HDCBS was selected for use in the final reagent composition as it produced the best sensitivity.

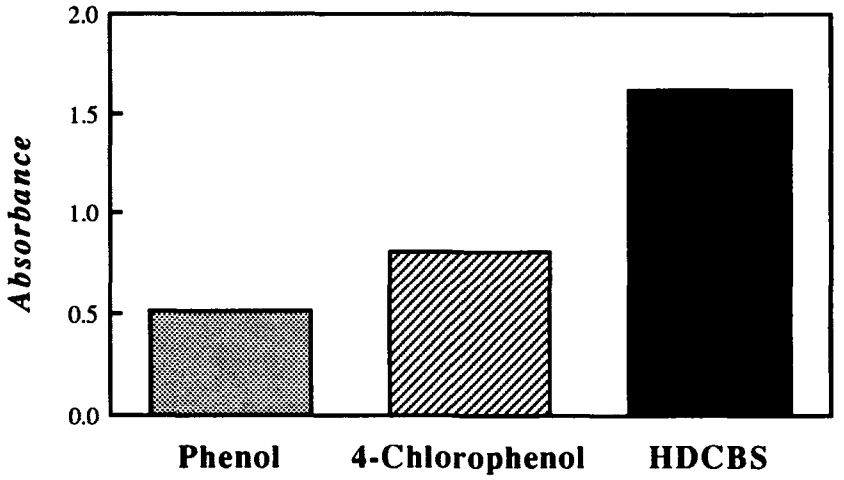


Figure 3. Effect of optional substrates in PG reagent II on the absorbance of a 125 µmol/L PG standard.

Glycerol-3-Phosphate Oxidase Activity

The final phase in developing the reagent composition was to determine the minimum amount of glycerol-3-phosphate oxidase required to drive the reaction to completion in less than 15 minutes. At the levels used by Artiss et al. (13) for all enzymes, the reaction reached its endpoint within 10 minutes. Glycerol-3-phosphate oxidase was selected for optimization because a cost analysis of the enzymes showed it to be the most expensive of the reagents. Table 2 shows the cost per unit and cost per mL of PG reagent for each

Table 2. Enzyme cost analysis.

Enzyme	U/mL	Cost ($) per unit (U)	Cost ($) PG Reagent per mL
Phospholipase D	80	0.005	0.40
Glycerokinase	1	0.14	0.14
Glycerol-3-phosphate oxidase	5	0.37	1.85
Peroxidase	2	0.002	0.004

enzyme used when following the procedure developed by Artiss et al (13). To reduce the $1.86 per mL cost for glycerol-3-phosphate oxidase, various amounts of the enzyme were added to the PG reagent . Testing was then performed on the DU-70 spectrophotometer by measuring the absorbance at 510 nm 15 minutes after combining reagent and sample. A 125 µmol/L glycerol standard was used to test the effect of each PG reagent, and the results compared to determine the minimum amount required for the reaction completion at 15 minutes. As shown in Figure 4, 3.0 U/mL was the lowest amount that still ensured the reaction would achieve its endpoint. The result was a savings of $ 0.75 per mL of reagent by reducing glycerol-3-phosphate oxidase to 3 U/mL without altering the time required to reach the reaction's endpoint.

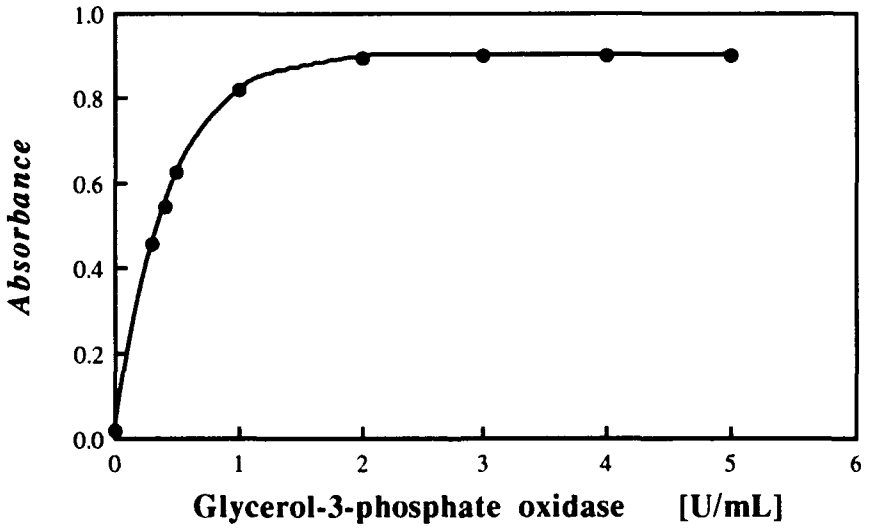


Figure 4. Optimization of glycerol-3-phosphate oxidase.
Effect of changing the amount of glycerol-3-phosphate oxidase in PG reagent using a 125 µmol/L glycerol standard.

Combination of Enzymatic Reagent

In deciding how to combine the enzymes and substrate into the reagent cocktail, several options were available. Initial tests were performed using a single reagent similar to Artiss et al. (13), but the reaction was too slow. The next group of tests used a two step reagent (PG reagent I and PG reagent II) similar to Muneshige et al. (14) and color development was more rapid. To further evaluate the two part reagent scheme, an evaluation was performed comparing the absorbance of a 125 μmol/L phosphatidylglycerol standard. Four different reagent combinations were tested with the contents of each PG reagent I and II listed in Table 3. Each reagent combination was tested observing the change of absorbances verses time at 510 nm for 15 minutes (900 sec) following addition of PG reagent II. Results are shown in Figure 5 based on the enzyme content of PG reagent I. The combination of enzyme and substrate used for PG reagent combination 3 (see Table 3) resulted in the most rapid kinetics. While PG reagent combinations 1 and 2 also gave good results, PG reagent 3 was chosen for further use as it reached endpoint sooner. PG reagent combination 4 results demonstrated an interference when all enzymes and substrate were combined together. As this combination was not used, investigation of the interference was not performed.

Glycerol Blank

One additional modification to the reagent was made prior to comparing the enzymatic assay to established indicators of fetal lung maturity. Previous studies (9,28,29) showed that endogenous glycerol present in amniotic fluid could falsely elevate the results of enzymatic phosphatidylglycerol determination. To prevent interference, endogenous glycerol must be removed from the assay by physical or chemical methods as glycerol is an intermediate in the enzymatic reaction. While phospholipid separation methods used to prepare amniotic fluid remove most of the glycerol, small amounts remained that could affect results. So, to minimize this problem, a glycerol blank was developed similar to that

Table 3. Contents of PG reagents I and II.

	PG Reagent Combinations:							
	1		2		3		4	
	PG I	PG II	PG I	PG II	PG I	PG II	PG I	PG II
PL-D	+	−	+	−	+	−	+	−
GK	−	+	+	−	+	−	+	−
GPO	−	+	−	+	+	−	+	−
PO	−	+	−	+	−	+	+	−
ATP	−	+	+	−	+	−	+	−
$CaCl_2$	+	−	+	−	+	−	+	−
$MgCl_2$	−	+	+	−	+	−	+	−
4-AAP	−	+	−	+	−	+	+	−
HDCBS	−	+	−	+	−	+	+	−

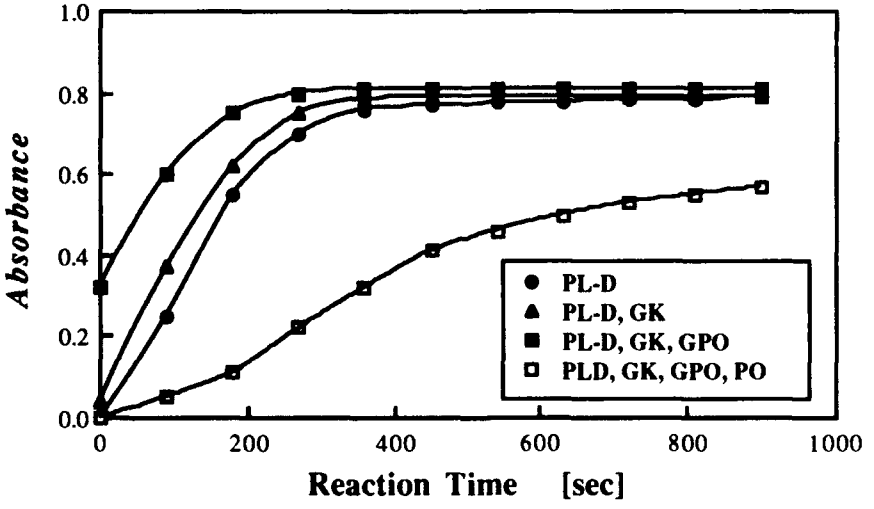


Figure 5. Enzymatic reagent combination review.
Studied the best combination of enzymes to optimize the overall reaction using a 125 µmol/L PG standard. The legend indicates the enzyme contents of PG reagent I with the complement of enzymes in reagent II.

used by Chapman et al. (15) for each sample by altering PG reagent I. PG reagent I was subdivided, one portion without phospholipase-D (designated PG reagent A) and one portion with phospholipase-D (designated PG reagent B). PG reagent II was redesignated PG reagent C, but remained unchanged in composition.

Using a split sample, PG reagent A was added to one portion and PG reagent B to the other. Following the initial 15 minute incubation, PG reagent C was added to both portions of the sample. After measuring the absorbances, PG reagent A, which represents the endogenous glycerol, was subtracted from PG reagent B, representing the combined endogenous glycerol and phosphatidylglycerol content of the sample. The resulting absorbance difference reflected the phosphatidylglycerol concentration of the sample. Also, as two portions from the same sample were used in blanking for glycerol and detecting phosphatidylglycerol, absorbance increases caused by turbidity and fluid coloration were corrected. This process was tested by spiking 50 µmol/L phosphatidylglycerol standards with 500 µmol/L glycerol standards. Using two samples of 50 µmol/L phosphatidylglycerol standard, one had glycerol added while the other had an equal volume of triton buffer added. Following enzymatic testing, the result showed no detectable increase in phosphatidylglycerol caused by the presence of glycerol. While glycerol concentrations are approximately 60 µmol/L in amniotic fluid, the phospholipid separation procedures appears to removed the majority of endogenous glycerol before testing. In most cases, following phospholipid separation, endogenous glycerol concentration detected using PG reagent A was < 1.5 µmol/L. An amount this low does not affect the results when using the glycerol blanking step.

Linearity Determination

Using phosphatidylglycerol standards of 0 - 250 µmol/L, linearity was verified for the proposed enzymatic procedure. The linear regression for the line represented by the standards is shown in Figure 6. With a correlation coefficient of 0.999, the plot of the

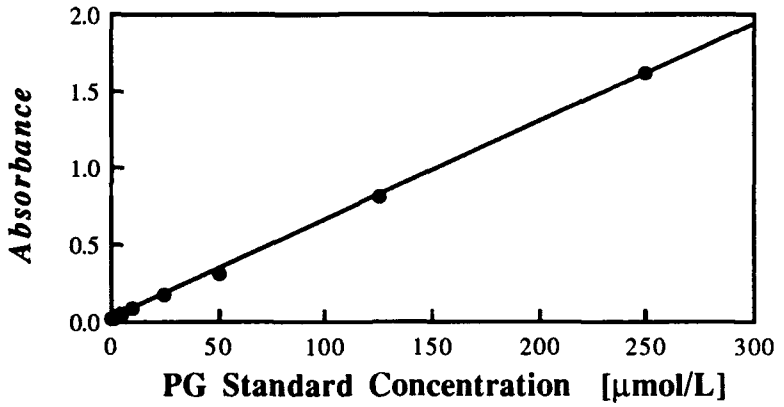


Figure 6. Linearity of aqueous PG standards.
Performed using PG standards in triton buffer and the enzymatic procedure without sample concentration. Linear regression for the line was:
$y = 0.0064x + 0.013$ and $r = .9999$.

standards formed a straight line and followed Beer's law. With linearity established, standard curves performed for tests run during the evaluation phase could be used to interpolate the concentration of unknown samples.

Interference Study

Phosphatidylglycerol assays have shown to be unaffected by common amniotic fluid contaminates (3,16,26,27). To determine the effect of various contaminates and phospholipids on the enzymatic procedure, a study was developed to identify possible interferants. The study was conducted using pooled phosphatidylglycerol positive amniotic fluids. Samples were prepared by pelleting amniotic fluid at 10,000 x g for 20 minutes, then resuspending the pellet with either triton buffer or a solution containing the suspected interfering substance. Samples were then tested by the enzymatic procedure described in the materials and methods section. Interfering substances were divided into two groups: phospholipids and contaminates. The phospholipid group consisted of phosphatidylcholine, phosphatidylinositol, and phosphatidylethanolamine. The contaminated group consisted of whole blood, meconium, and bilirubin. Testing was

designed to identify significant differences between amniotic fluid pellets suspended in triton buffer and those suspended in an interferant solution.

Each of the substances tested were prepared in triton buffer to the following concentrations: phospholipids (PC, PI, PE), 200 μmol/L; whole blood, 2 μL/mL; meconium, 5 mg/mL; and bilirubin, 0.1 mg/100 mL. Testing was performed in triplicate for each sample type. Following testing and interpolation of the phosphatidylglycerol content, paired t-tests were performed using the hypothesis: triton pellet PG = interferant pellet PG. The results (Table 4) of each t-test showed there was no significant difference ($P > 0.01$) between the triton buffer samples and interferant solution samples. At the interferant concentrations used, the phosphatidylglycerol assay is not affected.

Phosphatidylglycerol Recovery Study

To demonstrate the completeness of phospholipid separation methods used, several recovery studies were performed. Chloroform:methanol extraction was evaluated using both aqueous standards and amniotic fluids. Phosphatidylglycerol standards were

Table 4. Effects of possible interferants on phosphatidylglycerol determinations.

	PG Concentration (μmol/L)			
Interferant	^{W}/Interferant	$^{W}/_{O}$ Interferant	Difference	t-Test Probability alpha = 0.01
Blood	4.96	4.80	-0.16	0.0179
Meconium	4.62	4.80	0.18	0.0170
Bilirubin	4.74	4.80	0.06	0.0511
PC	7.57	7.53	-0.04	0.4130
PI	7.60	7.53	-0.07	0.0918
PE	7.37	7.53	0.16	0.0997

Interferant results are the average of three replicates.

extracted and the results compared to the expected concentrations. Amniotic fluid samples were split; to one sample triton buffer was added, to the other a solution containing phosphatidylglycerol. The amniotic fluids and standards were run in triplicate. Phosphatidylglycerol recovery was calculated using Equation 1. The results listed in Table 5 indicate an average recovery of 97.4%.

[1] Recovery = Concentration recovered/Concentration added x 100%

A traditional recovery study was not possible for centrifugation as amniotic fluid controls were not available and aqueous standards could not be pelleted. So, with extraction recovery determined to be 97.4%, centrifugation results were compared directly with extraction and the percent recovered a factor of extracted phosphatidylglycerol rather than total phosphatidylglycerol. Recovery percentages were calculated using Equation 2. Results from six amniotic fluids tested are listed in Table 6. The comparison shows the average recovery is 101.3%. While the data are insufficient to determine whether centrifugation separation is superior to extraction, the results indicate a slight improvement.

[2] Recovery = Pellet PG/Extracted PG x 100%

Comparison Study

Comparison of the proposed enzymatic procedure to established indicators of fetal lung maturity was essential to estimating its effectiveness. By demonstrating the correlation of the proposed method to the established indicators, its ability to predict fetal lung maturity could be inferred. The proposed enzymatic method was compared to: extracted enzymatic phosphatidylglycerol, lecithin/sphingomyelin ratio, and fluorescence polarization. Procedures used for all tests are described in the Materials and Methods section.

Table 5. Phosphatidylglycerol recovery by extraction

Sample Type	Concentration (μmol/L) Measured	Added	Recovered	Recovery (%)
Amniotic	8.4	5.0	4.79	95.8
Amniotic	11.8	5.0	4.75	95.0
Standard	35.2	5.0	4.96	99.1
Standard	64.9	5.0	4.99	99.8
			Mean	97.4

Data are the average of three replicates

Table 6. Phosphatidylglycerol recovery by centrifugation (10,000 x g)

Extracted PG (μmol/L)	Pellet PG (μmol/L)	Recovery (%)
5.6	5.7	101.8
7.4	7.6	102.7
14.3	13.9	97.2
6.6	6.9	104.6
4.3	4.2	97.7
2.7	2.8	103.7
	Mean	101.3

Recovery based on extract concentration rather than total phosphatidylglycerol concentration of amniotic fluid.

Phospholipid Separation: Pellet vs Extraction

The first step in evaluating the procedure was to compare the method of separating phospholipids from amniotic fluid. As previously stated, chloroform:methanol extraction has been advocated for the preparation of amniotic fluid prior to enzymatic testing (13,14,29). The proposed centrifugation method was compared directly to extraction to determine how closely the two methods correlate. Using two aliquots of the same amniotic fluid, phospholipids were separated by both extraction and centrifugation and then tested enzymatically. The result for the chloroform:methanol extraction aliquot, designated extract PG, and the centrifuged 10,000 x g pellet aliquot, designated pellet PG, are shown in (Figure 7). The methods showed an excellent correlation. With a regression line equation of $y = 0.08 + 0.997x$ and correlation coefficient of 0.990, little difference was seen between the two methods for the 55 fluids. While it was hoped that centrifugation would provide increased phosphatidylglycerol concentrations, having results equivalent to extraction still improves separation by eliminating the time consuming and technically difficult organic extraction.

One problem initially observed with extraction separation was incomplete recovery of chloroform. Ideally, all the chloroform should be recovered as it contains the phospholipids. In reviewing the effect of chloroform recovery on phosphatidylglycerol concentration, it was noticed that recovering < 90% of the expected chloroform, lowered the final result as compared to pellet PG. Of the 55 samples tested, 15 (27%) had less than 2.7 mL of recovered chloroform. Of these 15 samples, 7 (47%) showed significantly reduced phosphatidylglycerol concentration compared to pellet PG. This observation gave additional support to replacing extraction with centrifugation for separating phospholipids prior to enzymatic testing.

Fluorescence Polarization vs Lecithin/Sphingomyelin Ratio

For the amniotic fluids used in the comparison study, fluorescence polarization and lecithin/sphingomyelin ratio results were compared. These two tests were selected as the

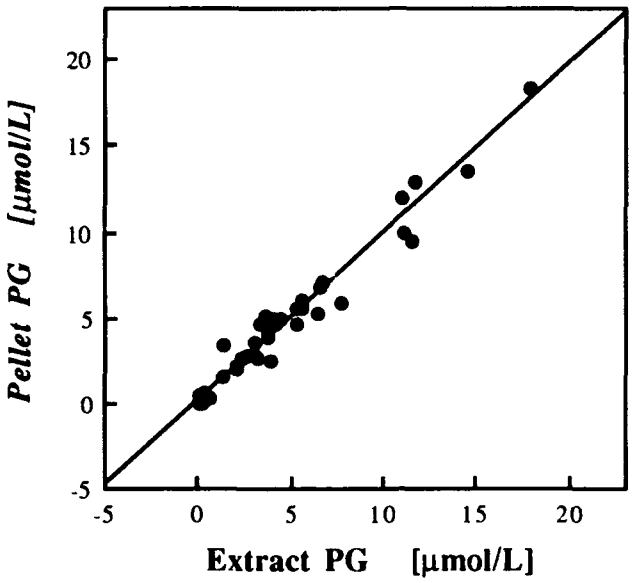


Figure 7. Comparison of phospholipid separation methods. Samples were split and separated by 10,000 x g centrifugation and chloroform:methanol extraction then tested using the enzymatic PG procedure.

lecithin/sphingomyelin ratio is considered the "gold standard" for fetal lung maturity testing by many clinicians and fluorescence polarization has demonstrated an ability to predict fetal lung maturity. Figure 8 shows the correlation between fluorescence polarization and the lecithin/sphingomyelin ratio. A correlation coefficient of -0.783 (n=101) agrees with studies by Foerder et al. (23) and Ashwood et al. (24) that reported correlation coefficients of -0.85 and -0.77 respectively. By using these tests as comparison standards, the utility of enzymatic phosphatidylglycerol to predict fetal lung maturity can be inferred.

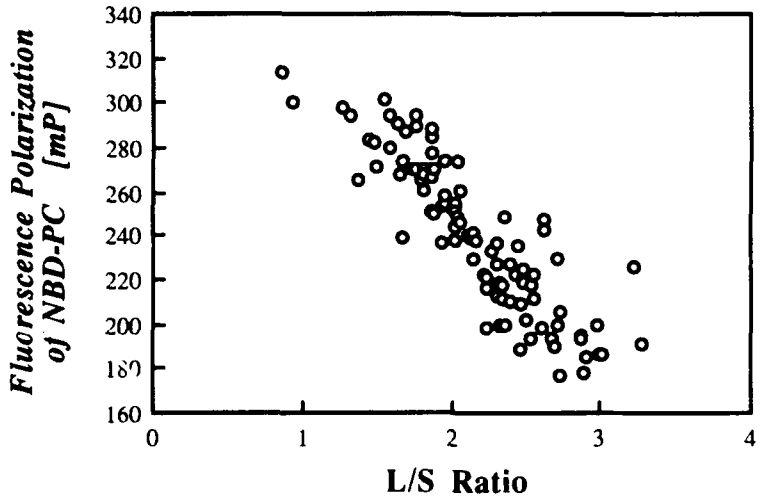


Figure 8. Comparision of polarization and L/S ratio data. Testing performed at ARUP Laboratories, Research Park, Salt Lake City, UT, using procedures described in the methods section.

Pellet Phosphatidylglycerol vs Lecithin/Sphingomyelin Ratio and Thin-Layer Chromatography Phosphatidylglycerol

This was the primary comparison to determine how effectively enzymatic phosphatidylglycerol would predict fetal lung maturity. Hallman et al. (7) showed that as the lecithin/sphingomyelin ratio exceeds 2.1, the phosphatidylglycerol result is usually positive. Being able to demonstrate that the proposed enzymatic pellet PG correlates with the lecithin/sphingomyelin ratio will infer its clinical usefulness. One hundred and one amniotic fluid samples were used in the comparison; lecithin/sphingomyelin ratio varied from 0.87 to 3.28 and pellet PG varied from 0.0 to 18.3 µmol/L. Also included with this comparison were the results for phosphatidylglycerol determined by thin-layer chromatography (TLC-PG). TLC-PG is the standard method for determining phosphatidylglycerol and provides qualitative results of negative, weak positive, and positive. The pellet PG results correlated well with the lecithin/sphingomyelin ratio ($r = 0.736$)(Figure 9). Using quantitative designations for phosphatidylglycerol suggested by Farquharson et al. (9) (negative < 0.5 µmol/L, weak positive 0.5-1.5 µmol/L,

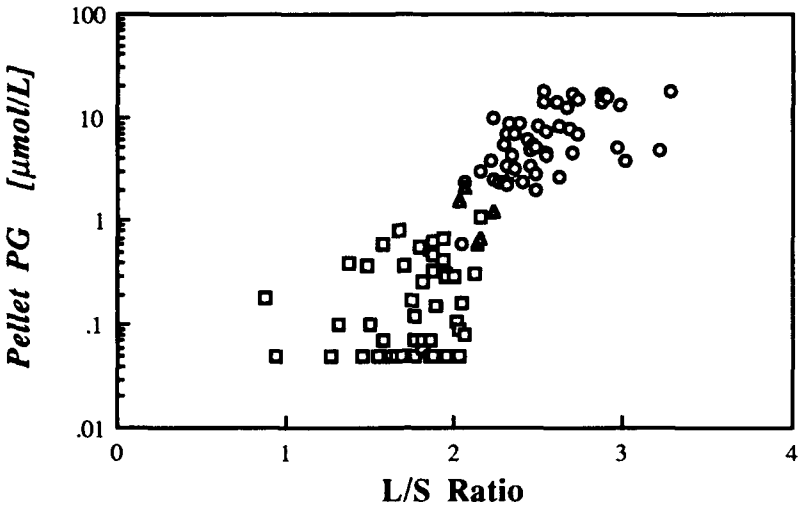


Figure 9. Comparison of pellet PG, L/S ratio, and TLC PG results.
Shows the relationship between pellet PG and TLC PG compared to the L/S ratio. Open squares represent negative TLC PG, open triangles represent weak positive TLC PG, and open circles represent positive TLC PG. Results of 0.05 indicate ≤ 0.05µmol/L.

positive > 1.5 µmol/L), TLC-PG and pellet PG were highly correlated. Below 0.5 µmol/L, all TLC-PG results were negative and above 1.5 µmol/L phosphatidylglycerol was always detected by TLC-PG.

For this comparison, the lecithin/sphingomyelin ratio was 2.2 before the phosphatidylglycerol concentration indicated maturity using Farquharson's criteria. While it was hoped that phosphatidylglycerol would infer maturity earlier than lecithin/sphingomyelin, the results showed phosphatidylglycerol lagged behind but did correlate with the lecithin/sphingomyelin ratio. Each positive phosphatidylglycerol result (>1.5 µmol/L) correlated with a lecithin/sphingomyelin ratio of ≥ 2.1. This finding supports the belief that a positive phosphatidylglycerol result can indicate fetal lung maturity without validation by other tests.

Pellet Phosphatidylglycerol vs Fluorescence Polarization

Studies by Hamilton et al. (11) and Simon et al. (30) considered the comparison of phosphatidylglycerol and fluorescence polarization. In both studies, phosphatidylglycerol was evaluated qualitatively as part of the fluorescence polarization and lecithin/sphingomyelin ratio comparison. Being able to correlate pellet PG with fluorescence polarization would provide additional evidence that pellet PG can effectively predict fetal lung maturity compared to established methods.

Evaluated for this comparison were 128 amniotic fluid samples, 101 of these samples were also used in the lecithin/sphingomyelin comparison. As was demonstrated with the lecithin/sphingomyelin ratio comparison, pellet PG lagged slightly behind a mature fluorescence polarization result. Referring to Figure 10, a fluorescence polarization value < 260 mP indicates fetal lung maturity, but the pellet PG was not consistently > 1.5 µmol/L until the fluorescence polarization was < 238 mP. Of 68 samples with fluorescence polarization < 238 mP, only 2 (2.9%) had a low phosphatidylglycerol result (< 1.5 µmol/L). Also, of 73 samples positive for phosphatidylglycerol, all 73 were predicted to be mature by fluorescence polarization. Overall, pellet PG was well correlated with fluorescence polarization ($r = -0.742$). Once again, the results showed strong evidence that enzymatic pellet PG may predict fetal lung maturity.

Reproducibility Study

The reproducibility of the enzymatic procedure was evaluated by performing a series of analyses on pooled amniotic fluid controls. Three controls were prepared from amniotic fluid with fluorescence polarization in the range of 180 to 190 mP, 220 to 230 mP, and 260 to 300 mP (control I, control II, and control III respectively). Once pooled, controls were separated into 3 mL aliquots and frozen at –60°C until tested. Over a 9 day period, 20 runs were performed using centrifugation (10,000 x g) separation with enzymatic testing. Each run included three controls (levels I, II, III), three phosphatidylglycerol standards (0, 30, 60 µmol/L), and amniotic fluid samples with unknown phosphatidylglycerol concentration.

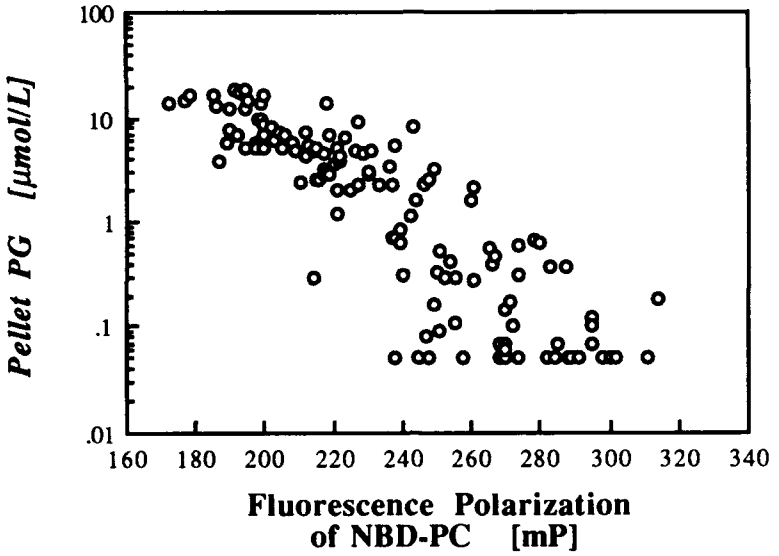


Figure 10. Comparison of pellet PG and polarization
Shows the relationship between fluorescence polarization and centrifuged pellets (10,000 x g) enzymatically tested for phosphatidylglycerol. Results of 0.05 indicate ≤ 0.05 µmol/L.

The results, shown in Table 7, examined mean, standard deviation, and coefficient of variation for each control as day-to-day data and for the study in total. A Levey-Jenning plot is shown in Figure 11. Assuming that the minimum detectable limit is 2 SD's from zero, this assay can detect phosphatidylglycerol concentrations down to 0.18 µmol/L. The results indicate that the proposed method's reproducibility will allow detection of small concentrations of phosphatidylglycerol with good precision.

Table 7. Reproducibility study for pooled amniotic fluid

	Day-to-Day	Total
Control I		
Mean, µmol/L	10.70	10.74
SD, µmol/L	0.32	0.28
CV, %	2.96	2.62
n	9	20
Control II		
Mean, µmol/L	3.91	3.93
SD, µmol/L	0.23	0.22
CV, %	5.92	5.67
n	9	20
Control III		
Mean, µmol/L	0.31	0.32
SD, µmol/L	0.10	0.09
CV, %	32.84	27.50
n	9	20

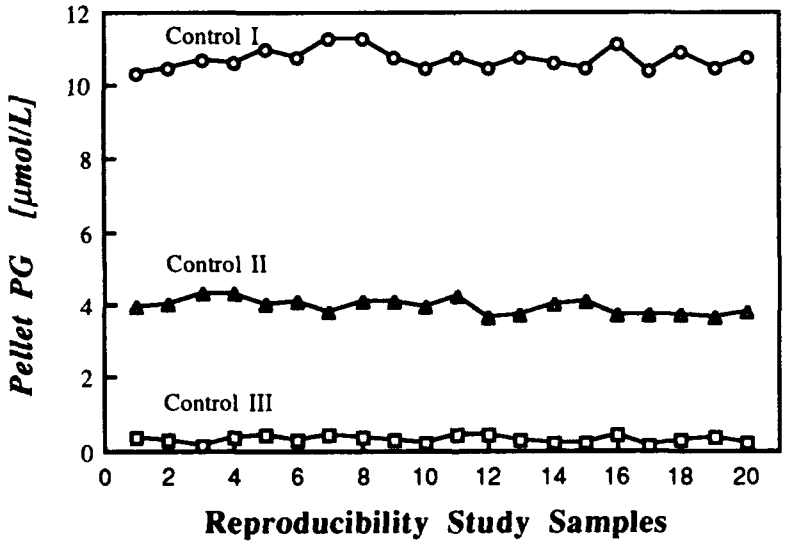


Figure 11. Reproducibility of pooled amniotic fluid.
20 samples run to determine reproducability of the enzymatic phosphatidylglycerol assay. Testing performed over a nine day peroid.

DISCUSSION

This study demonstrated the ability of an enzymatic phosphatidylglycerol assay to accurately correlate with established fetal lung maturity indices. A simplified procedure was created by designing a technique that uniquely combined a centrifugation method for phospholipid separation with enzymatic quantitation. Comparison of the proposed method with established methods showed a strong correlation between phosphatidylglycerol concentration and other tests for fetal lung maturity.

Phosphatidylglycerol indicates lung maturity when present. Problems with current testing methods include technical difficulty, time involved, and subjectiveness of result interpretation. The proposed method addresses these concerns by eliminating the organic phospholipid extraction and identification of spots on a chromatography plate. By using an enzymatic method, the results can be quantified to improve the correlation between phosphatidylglycerol concentration and stage of fetal lung maturation.

Initial method development involved selection of the reagents for the enzymatic reaction. Optimizing the composition and amounts resulted in a reagent that provided stable color development and good sensitivity. Using the maturity concentration (> 1.5 μmol/L) established by Farquharson et al. (9) as a starting point, a minimum detectable concentration of ≤ 1 μmol/L was desired. By using aqueous standards and pooled amniotic fluid controls, the procedure was shown to be linear from 0 - 250 μmol/L and have a minimum detectable amount of 0.18 μmol/L.

Farquharson et al. (9) reported an enzymatic method that could detect phosphatidylglycerol levels of ≥ 0.5 μmol/L with a maturity cutoff of > 1.5 μmol/L. Using this as a reference point, the proposed assay appears able to detect

phosphatidylglycerol three times lower. This ability to detect levels of ≥ 0.18 μmol/L means a better picture of phosphatidylglycerol's role in the fetal lung maturation process can be obtained. Current literature indicates that phosphatidylglycerol appears after 35 weeks gestation (7). This data was collected using methods unable to detect phosphatidylglycerol at concentrations < 0.5 μmol/L. By lowering the detection limit to 0.18 μmol/L, phosphatidylglycerol may be detected earlier in gestation (30 to 32 weeks). If future studies show the presence of low concentrations (< 0.5 μmol/L) of phosphatidylglycerol indicates lung maturity , then this method will provide more information to the clinician dealing with patients having premature labor or fetal distress. Knowing the risk of lung immaturity at each concentration of phosphatidylglycerol can help the clinicians manage the pregnancy.

Separation of the phospholipids by centrifugation provides a unique variation to the proposed enzymatic procedure. Chloroform:methanol extraction is the standard method for separating phospholipids from amniotic fluid. Centrifugation at 10,000 x g correlated well with extraction ($y = 0.08 + 0.997x$, $r = 0.990$) and is technically less difficult to perform. While it was hoped that centrifugation would provide a significant increase in measurable phosphatidylglycerol, no difference was found. Taking advantage of the simplified centrifugation method, the proposed method requires less time to separate phospholipids, approximately 20 minutes, without sacrificing quantity recovered. Centrifugation and extraction both allowed the phospholipids to be concentrated. With maturity onset believed to be around 1.5 μmol/L (9), concentrating the phospholipids would allow better detection of phosphatidylglycerol in transitional and immature fluids. This improvement allows for a better assessment of phosphatidylglycerol's role in lung development prior to maturity.

Another benefit of the proposed method's replacement of organic extraction and thin-layer chromatography, this test is suitable for small labs currently unable to perform fetal lung maturity testing. Technical manpower and resources in most small facilities are limited. The 3 hours required to perform a lecithin/sphingomyelin ratio is not practical and

sending samples to a reference laboratory presents major time obstacles to the clinician needing rapid results to decide on proper management for a complicated pregnancy. Requiring less than 1.5 hours and basic equipment, the proposed procedure can provide in-house testing at even the smallest of laboratories. Once the amniotic fluid is centrifuged and the pellet reconstituted, the procedure is as simple to perform as glucose, bilirubin, or triglyceride determinations, with reagent cost of only $0.31 per reaction tube. Also, interpretation of the results requires less subjectivity and judgement as the results are calculated directly from a standard curve. Automation of the procedure could reduce the completion time to < 1 hour with actual technologist time of approximately 30 minutes. Performing the test in the same facility where the patient is located, can improve the quality of care by quickly providing the clinician valuable information for the management of premature labor and fetal distress.

Endogenous glycerol can significantly interfere in enzymatic phosphatidylglycerol because glycerol is an intermediate in the enzymatic reaction sequence. Herold and Reed (28) showed endogenous glycerol must be removed to accurately measure phosphatidylglycerol. Centrifugation removed the majority of the glycerol when the supernate was decanted. To eliminate any residual glycerol, a reagent blank was incorporated that corrected for both the remaining glycerol and turbidity of the sample. PG-Numeric™ (15) also uses a glycerol blank, but it blanks the entire amount of glycerol in the amniotic fluid. This causes an elevated background absorbance that makes it difficult to detect low concentrations of phosphatidylglycerol. The result appears to be a reduction in net absorbance (PG reaction tube – glycerol blank) and sensitivity of the PG-Numeric™ procedure when compared to the proposed method. A 10 µmol/L phosphatidylglycerol standard produces an net absorbance of 0.0066 with PG-Numeric™ as compared with approximately 0.1900 with the proposed method. This represents a 29-fold improvement of net absorbance . The overall reaction scheme for PG-Numeric™ and the proposed method are similar, but the improvement provided by centrifugal separation and

concentration of the phospholipids has greatly enhanced the sensitivity. Improving the sensitivity will benefit both the clinician and researcher. The clinician will have a better quantitative gauge of maturity status, and the researcher can map the appearance of phosphatidylglycerol and determine if phosphatidylglycerol's role in lung maturity is concentration dependent.

Clinical evaluation of the method was performed by comparing it to established indicators of fetal lung maturity. Lecithin/sphingomyelin ratio and fluorescence polarization were used as the established indicators. Comparison of enzymatically tested samples showed good correlation with lecithin/sphingomyelin ratio ($r = 0.736$; $n = 101$) and fluorescence polarization ($r = -0.742$; $n = 128$). Using Farquharson's suggested maturity ranges in the comparisons, the enzymatic phosphatidylglycerol results inferred maturity later in gestation than the comparison methods. However, in all cases, when phosphatidylglycerol inferred maturity, so did the comparison method.

A problem with using phosphatidylglycerol to predict fetal lung maturity results from not having an established range for comparison. While this study was not designed to establish an expected range, by reviewing the relationship between phosphatidylglycerol, lecithin/sphingomyelin ratio, and fluorescence polarization, an expected range can be estimated but not verified. In reviewing the correlation between phosphatidylglycerol comparison methods, both linear and nonlinear logarithmic polynomial correlations (first, second, and third order) were considered. For both comparison methods, second and third order polynomial correlations failed to provide significant improvement in correlation over first order polynomial (log-linear) correlation so, the first order correlation was used to estimate an expected range.

Reviewing the scatter of values in Figures 9 and 10, the data show a gradual exponential increase as the lecithin/sphingomyelin ratio increases or fluorescence polarization decreases. This indicates the possibility for establishing standard ranges in the categories of immature, transitional, and mature. Data compiled during this study show

potential for the results to be accurately differentiated by ± 0.1 µmol/L. With that level of precision, maturity and immaturity can be predicted with more confidence .

Both the lecithin/sphingomyelin ratio and fluorescence polarization comparisons showed lower phosphatidylglycerol concentrations at maturity onset than previously reported (9,15). The lecithin/sphingomyelin ratio comparison yielded a regression line of $y = -3.21 + 1.49x$. For fluorescence polarization, the equation was $y = 5.12 - 0.021x$. For both equations; $y = \log_{10}$ (PG) and x = comparison method result. Using the following reference ranges for lecithin/sphingomyelin (L/S) and fluorescence polarization (FPol), the corresponding phosphatidylglycerol ranges were estimated from the regression lines: lecithin/sphingomyelin ratio - immature < 1.5, transitional 1.5 to 2.0, mature > 2.0; fluorescence polarization - immature > 290 mP, transitional 260 to 290 mP, mature < 260 mP. A lecithin/sphingomyelin ratio of 1.5 and 2.0 resulted in phosphatidylglycerol values of 0.1 and 0.6 µmol/L respectively. This would suggest the following phosphatidylglycerol ranges: immature < 0.1 µmol/L, transitional 0.1 to 0.6 µmol/L, and mature > 0.6 µmol/L. For fluorescence polarization, results of 290 and 260 mP resulted in phosphatidylglycerol values of 0.1 and 0.5 µmol/L respectively, Using this comparison, the suggested phosphatidylglycerol ranges would be: immature < 0.1 µmol/L, transitional 0.1 to 0.5 µmol/L, and mature > 0.5 µmol/L. While phosphatidylglycerol ranges are only an observation based on correlation with comparison methods, the lower values may indicate an improved sensitivity for enzymatic testing.

Using the above reference ranges, Table 8 shows the comparison of the proposed method's inferred predictions versus lecithin/sphingomyelin. Fifty-four (86%) of the 63 samples mature by the lecithin/sphingomyelin ratio were also mature using the estimated phosphatidylglycerol ranges. Table 9 repeats the comparison by replacing the lecithin/sphingomyelin ratio with fluorescence polarization. For the 93 samples mature by fluorescence polarization, 79 (84%) were also mature using the estimated phosphatidylglycerol ranges. A review of immaturity showed similar results with 36

Table 8. Comparison of maturity predictions:
Proposed PG vs L/S ratio

	Immature (PG < 0.6 µmol/L)	Mature (PG > 0.6 µmol/L)	Total
Immature (L/S < 2)	36	2	38
Mature (L/S >2)	9	54	63
Total	45	56	101

(95%) of the 38 samples immature by the lecithin sphingomyelin ratio also immature by the estimated ranges (Table 8). Likewise, 30 (86%) of 35 immature fluorescence polarization samples were estimated as immature based on phosphatidylglycerol concentration (Table 9). For these comparisons, immature and transitional results were grouped together as immature. The results indicate a potential for accurate prediction of maturity and immaturity. While sensitivity needs to be improved, it can be accomplished by adjusting

Table 9. Comparison of maturity predictions:
Proposed PG vs polarization

	Immature (PG < 0.5 µmol/L)	Mature (PG > 0.5 µmol/L)	Total
Immature (FPol > 260)	30	5	35
Mature (FPol < 260)	14	79	93
Total	44	84	128

the expected ranges based on additional testing and fetal outcome studies. While additional studies are required to document the diagnostic value of enzymatic phosphatidylglycerol, these initial reviews indicate the procedure's potential to predict fetal lung maturity and immaturity with precision equal to lecithin/sphingomyelin ratio and fluorescence polarization.

Interference, a problem encountered by both the lecithin/sphingomyelin ratio and fluorescence polarization , was evaluated and found not to significantly affect phosphatidylglycerol results using the proposed method. Tests for the effects of blood, meconium, bilirubin, and other phospholipids at concentrations 10 times that possibly found in amniotic fluid did not indicate interference of phosphatidylglycerol results. This evaluation agrees with previous reports (16,26,27) by demonstrating phosphatidylglycerol is suitable for samples contaminated with blood, meconium, or bilirubin.

To determine the enzymatic procedure's reproducibility, three levels of pooled amniotic fluid controls were tested. Over a 9 day period, 20 runs were performed. Each run included one sample of control I, II, and III with means of 10.70, 3.61, and 0.31 μmol/L respectively. The results showed good reproducibility for all 3 levels. With daily coefficient of variations (CV) of 3.0% , 5.9%, and 32.8% for control I, II, and III respectively, the proposed phosphatidylglycerol method showed improvement over other enzymatic procedures. To determine how well the proposed phosphatidylglycerol method's reproducibility compared with other enzymatic methods, data from Artiss et al. (13), Chapman et al. (15), and Farquharson et al. (9) were compared. Table 10 shows the concentration (μmol/L) of each control and the corresponding CV (%). Although concentration of controls were slightly different than those used by Artiss et al. (13), Chapman et al. (15), and Farquharson et al. (9), the results showed improved reproducibility for similar concentrations. Increased precision is important in validating a method used in clinical diagnosis. Tests giving sporadic results are less reliable indicators

of physiological conditions. As indicated by this study, enzymatic testing for phosphatidylglycerol concentration showed better reproducibility than any other phosphatidylglycerol method.

To further demonstrate enzymatic analysis of a 10,000 x g lamellar body pellet is an improved phosphatidylglycerol method, an attempt was made to evaluate the maturity cut-off of each method. Artiss did not propose a range, Farquharson suggested a range of immature < 0.5 μmol/L, transitional 0.5 to 1.5 μmol/L, and mature > 1.5 μmol/L, but only Chapman reports an established range of maturity ≥ 2.5 μmol/L. As suggested earlier, a value of 0.6 μmol/L using the proposed method may indicate maturity, but even if additional studies increase the cut-off to 1.0 μmol/L, that still represents an improvement of at least 30% over other enzymatic phosphatidylglycerol methods. It would appear, based on estimating phosphatidylglycerol values from the lecithin/sphingomyelin ratio and fluorescence polarization, the proposed method may indicate maturity before other enzymatic phosphatidylglycerol methods.

While additional study is needed to determine if enzymatic phosphatidylglycerol testing of lamellar body pellets is an improved method, the data from this study show it has the potential to accurately indicate fetal lung maturity. To further evaluate the effectiveness of

Table 10. Comparison of reproducibility between enzymatic methods.

Phosphatidylglycerol, μmol/L (CV, %)			
Artiss (13)	Chapman (15)	Farquharson (9)	Proposed
31.2 (6.1%)	----------	----------	----------
6.5 (12.5%)	7.28 (6.2%)	11.2 (3.5%)	10.70 (3.0%)
----------	2.55 (11.4%)	2.1 (15%)	3.91 (5.9%)
----------	----------	0.65 (46%)	0.31 (32.8%)

enzymatic phosphatidylglycerol to accurately predict maturity of the fetal lungs, an extensive retrospective or perspective study should be performed. This study should include: gestational age of the fetus at the time of amniotic fluid collection; outcome of infants born within 72 hours of specimen collection; maternal complications (diabetes, hypertension, prolonged rupture of membranes, etc.); and a comparison of enzymatic phosphatidylglycerol, lecithin/sphingomyelin ratio, and fluorescence polarization to fetal outcome. Amniotic fluids from newborns presenting symptoms of respiratory difficulty should compose a significant number of the total samples evaluated. The exact usefulness of the proposed enzymatic procedure can not be determined until a study of this magnitude is completed. Being able to directly correlate enzymatic phosphatidylglycerol results with clinical outcomes will define the degree of accuracy for predicting maturity and immaturity.

By combining centrifugation (10,000 x g) for phospholipid separation with enzymatic phosphatidylglycerol determination, an accurate, reliable, and simplified procedure for measuring phosphatidylglycerol is obtained. Suitable for small laboratories, the proposed phosphatidylglycerol method can provide quick and accurate results to clinicians on fetal lung maturity. Clinical management will be improved by quantitating the maturation process rather than just indicating maturity or immaturity. As phosphatidylglycerol can indicate fetal lung maturity, this method may provide an alternative to current fetal lung maturity tests. With good correlation to established methods, phosphatidylglycerol has shown elevated concentrations when maturity is indicated and decreased concentrations when immaturity is suspected. Enzymatic analysis of a 10,000 x g amniotic fluid pellet has the potential to become a valuable test for predicting fetal lung maturity.

REFERENCES

1. Nelson GH. Pulmonary Development. New York: Marcel Dekker, 1985:349-60.

2. Stern L. Hyaline membrane disease. Orlando: Grune and Statton, 1984:35-8, 63-113.

3. Spillman T, Cotton DB. Current perspectives in assessment of fetal pulmonary surfactant status with amniotic fluid. Crit Rev Clin Lab Sci 1989; 27:341-89.

4. Chapman JF, Herbert WNP. Current methods for evaluating fetal lung maturity. Lab Med 1986; 17:597-602.

5. Gluck L, Kulovich MV, Borer RC, Brenner PH, Anderson GG, Spellacy WN. Diagnosis of the respiratory distress syndrome by amniocentesis. Am J Obstet Gynecol 1971; 109:440-45.

6. Spillman T, Cotton DB, Golunski E. Detection frequency by thin-layer chromatography of phosphatidylglycerol in amniotic fluid with clinically functional surfactant. Clin Chem 1988; 34:1976-82.

7. Hallman M, Kulovich M, Kirkpatrick E, Sugarman RG, Gluck L. Phosphatidylinositol and phosphatidylglycerol in amniotic fluid: Indicies of lung maturity. Am J Obstet Gynecol 1976; 125:613-17.

8. Coapman-Hankin RA, Kiechle FL, Epstein E, Artiss JD, Zak B. Three methods compared for determining phosphatidylglycerol in amniotic fluid. Clin Chem 1985; 31:1374-76.

9. Farquharson J, Jamieson CE, Paton RD, Black J, Logan RW. Quanitative determination of phosphatidylglycerol in amniotic fluid by enzymatic assay. Clin Chem Acta 1985; 152:55-61.

10. Eisenbrey AB, Epstein E, Zak B, McEnroe RJ, Artiss JD, Kiechle FL. Phosphatidylglycerol in amniotic fluid. Am J Clin Path 1989; 91:293-7.

11. Hamilton PR, Hauschild D, Broekhuizen FF, Beck RM. Comparison of lecithin:sphingomyelin ratio, fluorescence polarization, and phosphatidylglycerol in the amniotic fluid in the prediction of respiratory distress syndrome. Obstet Gynecol 1981; 57:295-300.

12. McCulloch JC, Mendelsohn D. Fetal lung maturity tests on the 10,000 x g pellet. Clin Chem Acta 1988; 172:47-56.

13. Artiss JD, McGowan MW, Strandbergh DR, Epstein E, Zak B. Enzymatic colorimetric determination of phosphatidylglycerol in amniotic fluid. Clin Chem 1984; 30:534-7.

14. Muneshige A, Okazaki T, Quirt JG, MacDonald PC, Nozaki M, Johnston JM. A rapid and specific enzymatic method for the quanitification of phosphatidylcholine, disaturated phosphatidylcholine, and phosphatidylglycerol in amniotic fluid. Am J Obstet Gynecol 1983; 145:474-80.

15. Chapman JF, Phillips JC, Rosenthal MA, Herbert WNP. Evaluation of the PG-numeric™ assay for semi-automated analysis for phosphatidylglycerol in amniotic fluid. Clin Chem 1990; 36:1974-77.

16. Strassner HT, Golde SH, Mosley GH, Platt LD. Effect of blood in amniotic fluid on the detection of phosphatidylglycerol. Am J Obstet Gynecol 1980; 138:697-702.

17. Pastorek JG, Lectellier RL, Gebbia K. Production of a phosphatidylglycerol-like substance by genital flora bacteria. Am J Obstet Gynecol 1988; 159:199-202.

18. Schumacher RE, Parisi VM, Steady HM, Tsao FHC. Bacteria causing false positive test for phosphatidylglycerol in amniotic fluid. Am J Obstet Gynecol 1985; 151:1067-8.

19. Oulton M. The role of centrifugation in the measurement of surfactant in amniotic fluid. Am J Obstet Gynecol 1979; 135:337-43.

20. Oulton M, Bent AE, Gray JH, Luther ER, Peddle LJ. Assessment of fetal pulmonary maturity by phospholipid analysis of amniotic fluid lamellar bodies. Am J Obstet Gynecol 1982; 142:684-91.

21. Ashwood ER, Chamberlain BA. Binding of fluorescent phosphatidylcholine in amniotic fluid. Obstet Gynecol 1988; 71:370-74.

22. Tait JF, Franklin RW, Simpson JB, Ashwood ER. Improved fluorescence polarization assay for use in evaluating fetal lung maturity. I. Development of the assay procedure. Clin Chem 1986; 32:248-54.

23. Foerder CA, Tait JF, Franklin RW, Ashwood ER. Improved fluorescence polarization for use in evaluating fetal lung maturity. II. Analytical evaluation and comparison with the lecithin/sphingomyelin ratio. Clin Chem 1986; 32:255-59.

24. Ashwood ER, Tait JF, Foerder CA, Franklin RW, Benedetti TJ. Improved fluorescence polarization assay for use in evaluating fetal lung maturity. III. Retrospective clinical evaluation and comparison to the lecithin/sphingomyelin ratio. Clin Chem 1986; 32:260-64.

25. Tait JF, Foerder CA, Ashwood ER, Benedetti TJ. Prospective clinical evaluation of an improved fluorescence polarization assay for predicting fetal lung maturity. Clin Chem 1987; 33:554-58.

26. Yambao TJ, Tawwater B, Chuachingco J, Carrillo A, Schwartz A. Effect of meconium on the detection of phosphatidylglycerol. Am J Obstet Gynecol 1984; 150:426-30.

27. Buhi WC, Spellacy WN. Effects of blood or meconium on the determination of amniotic fluid lecithin/sphingomyelin ratio. Am J Obstet Gynecol 1975; 121:321-23.

28. Herold DA, Reed AE. Interference by endogenous glycerol in an enzymatic assay of phosphatidylglycerol in amniotic fluid. Clin Chem 1988; 34:560-63.

29. Farquharson J, Jamieson EC, Berry E, Sutton A, Logan RW. Combined enzymatic assay of phosphatidylglycerol and phosphatidylcholine in amniotic fluid. Clin Chem Acta 1988; 171:175-86.

30. Simon NV, Williams GH, Fairbrother PF, Elser RC, Perkins RP. Prediction of fetal lung maturity by amniotic fluid fluorescence polarization, L:S ratio, and phosphatidylglycerol. Obstet Gynecol 1981; 57:295-300.